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Interrelations of Chemistry and Biotechnology—I†

BIOTRANSFORMATION—A USEFUL TOOL IN ORGANIC CHEMISTRY

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†The Commission has chosen this series with a view to intensify the interrelations of chemistry and biotechnology. By improving the knowledge of chemists in the field of biotechnology it is hoped to initiate more ideas for applying biological methods in chemistry, inspire more use of chemical knowledge in the biological sciences and help scientists in both fields to work closer together. In the articles in the series, to be published in this journal, outstanding experts in their respective fields will give (a) an overview on topics related to the practical use of biotechnological methods in organic chemistry, (b) an outlook on upcoming research topics, their impact on existing areas and their potential for future developments.

The Commission solicits comments as well as suggestions for future topics, and will aim to help in providing answers to any questions in this field.

Biotransformation – a useful tool in organic chemistry

Abstract - This review discusses the characteristics, the methodology and the applications of biotransformations in organic chemistry. Biotransformations have successfully been employed to introduce chiral centers, to resolve racemates, to convert a particular functional group among several groups with similar reactivities, to functionalize a nonactivated carbon regioselectively and to convert labile molecules due to the mild reaction conditions under which they take place. Design and realization of combined chemical/biotechnological syntheses requires a close interdisciplinary collaboration. Successful practical examples are discussed. In view of nature's immense reservoir of enzyme activities and the significant development in modern biotechnology, the application potential of biotransformations in organic chemistry is by far not yet exhausted.

INTRODUCTION

Chemical reactions catalyzed by microorganisms or by enzyme preparations derived from biomass are termed biotransformations.

With a multiplicity of constitutive or inducible enzymes, microorganisms are capable of performing a vast number of chemical reactions which are essential for maintaining the life functions of the cell, including growth and reproduction. In fact, there exists an enzyme catalyzed equivalent for almost every type of chemical reaction (ref. 1). Microorganisms employ such enzyme catalyzed reactions which are well organized in metabolic pathways for the degradation or synthesis of a great variety of chemical compounds. Nutrients are degraded in catabolic pathways yielding energy and small molecules as building blocks for anabolic metabolism. The energy provided by exothermic degradation steps is needed for the maintenance of viability and to support endothermic anabolic metabolism in which all the constituents needed for cell growth and propagation are synthesized.

More than 2000 enzymes have been catalogued till now (ref. 2). Each of them accepts a certain substrate and catalyzes a particular reaction which usually represents one step in a metabolic pathway. Besides their natural substrates, many enzymes also accept foreign, but structurally related compounds, and thus catalyze "unnatural" reactions with substrates supplied to the medium. Reaction products which are not further degraded accumulate in the medium, from where they can be isolated. In this case, the microorganism or one of its enzymes respectively, acts simply as a catalyst, mediating the so-called biotransformation of a substrate A yielding the product B. The scope of chemical reaction types mediated by microorganisms is shown in Table 1 (refs. 3,4).

TABLE 1. Classification of chemical reaction types catalyzed by enzymes

Oxidations	Hydroxylation, epoxidation, dehydrogenation of C-C bonds. Oxidation of alcohols and aldehydes, oxidative degradation of alkyl, carboxyalkyl or oxoalkyl chains, oxidative removal of substituents, oxidative deamination, oxidation of hetero-functions, oxidative ring fission.
Reductions	Reduction of organic acids, aldehydes, ketones and hydrogenation of C=C bonds, reduction of hetero-functions, dehydroxylation, reductive elimination of substituents.
Hydrolysis	Hydrolysis of esters, amines, amides, lactones, ethers, lactams etc. Hydration of C=C bonds and epoxides.
Condensation	Dehydration, O- and N-acylation, glycosidation, esterification, lactonization, amination.
Isomerization	Migration of double bonds or oxygen functions, racemization, rearrangements.
Formation of	C-C bonds or hetero-atom bonds.

Innumerable biotransformations have been reported and reviewed in the literature (refs. 1,4-14). Several of them have been applied successfully on a preparative or even an industrial scale as favourable alternatives to pure chemical methods or even as the unique access to certain useful products. Biotransformation technology has proven to be an additional useful tool in synthetic organic chemistry. In this paper, the advantages of biotransformations over chemical procedures will be reviewed and their application potential will be illustrated with selected practical examples.

CHARACTERISTICS OF BIOTRANSFORMATIONS

Enzymes are biocatalysts which accelerate reaction rate in the same way as chemical catalysts. The salient features by which biocatalysts are favourably distinguished from common chemical catalysts is their high specificity, not only with respect to the reaction they catalyze but also with respect to the structure and even the stereochemistry of the substrate they accept and the product they form. Furthermore, high reaction rates are obtained under mild reaction conditions due to a significant decrease of the activation energy of chemical reactions in the enzyme/substrate complex. The main advantages of biotransformations are summarized in the following:

Reaction specificity

The catalytic activity of an enzyme is usually limited to one single reaction type yielding a homogeneous product. This means that no side reactions (or by-products respectively) have to be expected as long as only one enzyme is involved in a biotransformation.

Stereospecificity

The reaction site of an enzyme represents a complex, three-dimensional and asymmetric environment which enables the enzyme to display high selectivity with respect to its substrate and even to distinguish between different stereochemical configurations of substrate molecules, e.g. between the enantiomers of a racemic substrate. Thus, enzymes are capable of resolving racemates by transforming exclusively or at least preferentially one of the existing enantiomers. - On the other hand, if an enzyme reaction gives rise to a new center of asymmetry, the stereochemical configuration of the substituents is usually steered in a way, that only one of the possible enantiomers is formed. The product is therefore optically active.

Regiospecificity

In general, an enzyme attacks its substrate also specifically with respect to the position where the reaction takes place. This holds true even if several groups of equivalent or similar reactivity are present in the substrate molecule.

Mild reaction conditions

The activation energy of chemical reactions is significantly lowered by enzyme/substrate interactions. Thus, enzymes display high catalytic activities even under mild reaction conditions, i.e. in aqueous media, at temperatures below 40° C, at pH values near neutrality and at normal pressure. Thus, the harsh and energy consuming reaction conditions used for chemical catalysis can be avoided and even labile molecules can be converted without undesired decomposition or other side reactions.

As it will be exemplified later, the favourable characteristics of biotransformations discussed above provide the opportunity to carry out certain reaction steps which can hardly be accomplished by chemical methods. However, it should also be mentioned that in comparison to chemical reactions the following disadvantages of biotransformations have to be taken into account: The necessary expenditures for the development of a biotransformation process including the product isolation are usually high; in most cases the reaction time is rather long, the substrate/product concentrations low and the stability of the biocatalyst limited. These features are responsible for the generally rather high costs of biotransformation steps.

METHODOLOGY OF BIOTRANSFORMATIONS

Various biotransformations have successfully been carried out with biocatalysts which are commercially available, such as several enzymes and Baker's yeast. A comprehensive review covering such biotransformations has recently been published by Jones (ref. 1). Certainly, it is worth establishing whether a desired reaction step can be mediated by a purchasable biocatalyst. However, the scope of commercially available biocatalysts is limited and in many cases it is necessary to search for a microorganism harbouring a tailor-made enzyme with which the desired reaction can be accomplished. This requires the skills of a micro-

biologist. In the following, the major techniques involved in the development of biotransformation processes will be briefly summarized. More detailed reviews on the methodology of biotransformations have been published by Leuenberger (ref. 3) and Goodhue (ref. 15).

The substrate

A prerequisite of a successful biotransformation is the contact between the substrate and the enzyme. The ideal substrate should be readily soluble in an aqueous medium and able to pass the cell membrane without exerting a toxic effect on the biocatalyst. It is usually fed to a culture of microorganisms neat or as a sterile concentrated solution. The optimal growth phase for feeding and optimal substrate concentration have both to be determined experimentally.

Substrates which are not readily soluble in an aqueous medium are dissolved in a relatively non-toxic, water-miscible solvent prior to their addition to the culture (e.g. methanol, ethanol, ethylene glycol, acetone, propylene glycol, dimethyl sulfoxide). Another possibility for enhancing solubility is to add an emulsifying agent.

Substrates which affect growth or viability of microorganisms are added to the culture only after completion of growth. They are preferably dispensed semi-continuously in small portions or continuously at a low rate, such that their concentration in the reaction medium remains low.

If a biotransformation of a selected substrate fails to give satisfactory results, a minor chemical modification of the substrate should be considered, e.g. addition, removal or variation of a protecting group or change of the oxidation state of a functional group. Such measures can improve the properties of the substrate, e.g. by increasing the solubility, by decreasing the toxicity, by improving the permeability through the cell membrane, by improving the affinity to the enzyme or by reducing the susceptibility to undesired side reactions.

Selection of the biocatalyst

Usually a microorganism capable of converting a substrate A into a desired product B has to be sought by means of a laborious screening where a broad variety of microorganisms (bacteria, actinomycetes, fungi, yeasts) are tested for their ability to perform the desired reaction with highest possible yield. For this purpose, pure cultures of microorganisms are incubated in a suitable nutrient medium. The compound to be converted is added during growth or after completion of growth and the incubation is continued for 1-7 days. Samples are removed and analyzed for product formation.

Pure cultures of microorganisms can be obtained from public culture collections or by isolation from natural sources, e.g. from soil samples. The probability of success can be increased if microorganisms are selected which are known (from personal experience or from the literature) to be capable of mediating the desired reaction type with structurally related compounds. Valuable sources for the identification of such biocatalytically active microorganisms have been compiled for various substrate classes (refs. 13,14), for steroids (ref. 7), for non-steroid cyclic compounds (ref. 4), for alkanes, acyclics and terpenes (ref. 8), for antibiotics (ref. 9), for alkaloids and nitrogenous xenobiotics (ref. 10), for bioactive compounds including prostaglandins (ref. 11) and for optically active amino acids and α-hydroxy acids (ref. 12).

Reaction systems

Various reaction systems differing in the conditions under which the biocatalytically driven reaction is run have been successfully operated, partly even on a technical scale:

Biotransformations with growing cultures The substrate is added to the growth medium at the time of inoculation or during a later stage of microbial growth; this means, that growth and biotransformation take place simultaneously. The most favourable moment for substrate addition has to be determined experimentally. The major advantage of this technique is its ease of handling, which makes it suitable for large series of screening experiments. Incubation times are relatively short and there is the potential for induction of the desired enzyme activity during growth in presence of the substrate. On the other hand, if the substrate exerts an inhibitory effect on microbial growth, addition has to be postponed until growth has ceased.

Biotransformations with previously grown cultures In this case, the processes of biomass (biocatalyst) propagation and biotransformation are separated. The microorganisms are first cultivated under optimized growth conditions and the biomass is then harvested by centrifugation or filtration and stored in the refrigerator (if necessary). As occasion demands, the appropriate amount of biomass is resuspended in a simple "transformation

medium" consisting usually of an aqueous buffer solution with optimized pH value containing the solubilized substrate. Sometimes it is advisable to also supply an easily metabolizable nutrient such as glucose, in order to retain viability and thereby biocatalytic activity of the cells as long as possible. Such a cell suspension is then incubated until maximum product yield is obtained. This technique offers the following advantages: growth and bioconversion can be independently optimized, growth-inhibitory effects of the substrate or product are eliminated, the cell (biocatalyst) concentration affording optimum bioconversion can be adjusted as desired, the risk of infection by contaminant microorganisms during the biotransformation is reduced and product isolation is facilitated due to the simple composition of the transformation medium.

Baker's yeast is a cheap, commercially available biomass which has been used successfully for many useful biotransformations, particularly for various stereospecific reductions (refs. 1, 16, 17).

Biotransformations with purified enzymes If the permeability of the substrate through the cell membrane is insufficient or if undesired side (or sequential) reactions take place due to the presence of other enzyme systems, it is necessary to conduct the biotransformation with a cell-free enzyme preparation or even with a purified enzyme. Modern techniques for enzyme purification have recently been summarized by Jakoby (ref. 18). In general, enzyme purification processes are rather tedious and expensive. The catalytic activity of many enzymes involved in application oriented reactions (such as oxidoreductases or kinases) depends on the availability of specific cofactors such as NAD(H), NADP(H), $FAD(H_2)$ or ADP/ATP. A stoichiometric cofactor consumption is out of the question because of excessive costs. Thus, methods for efficient cofactor regeneration have to be established in cofactor-requiring cell-free systems, while living cells are able to supply and recycle the necessary cofactors themselves. Although several useful methods regarding the regeneration of NAD(H), NADP(H) or ADP/ATP have been suggested (refs. 19-22), and successfully applied in some particular cases, cofactor regeneration in technical processes is generally still a problem. Thus, hydrolyzing enzymes (hydrolases) which do not need any cofactors have preferably been used so far.

Of several thousand known enzymes (ref. 2), a few hundred have become commercially available. A comprehensive review on the application of enzymes in organic syntheses with particular emphasis on commercially available enzymes has recently been published by Jones (ref. 1).

Biotransformations with immobilized cells or enzymes A vast number of techniques for cell and enzyme immobilization have been described and thoroughly reviewed in the literature (refs. 23-26). The immobilization methods can be classified as follows:

- Entrapment within the lattice of a crosslinked polymer matrix, e.g. polyacrylamide gel, alginate, κ-carrageenan, cellulose.
- Attachment to a water-insoluble carrier material (e.g. DEAE-cellulose, concanavalin A, ion-exchange resins) by physical adsorption or ionic or convalent binding.
- Aggregation by chemical or physical crosslinking with multifunctional agents, e.g. glutaraldehyde or poly(iminoethylene).

With immobilized cells or enzymes it is easy to remove the biocatalyst from the reaction broth and to use it repeatedly or to retain it within the reaction vessel and to run the biotransformation process continuously. Furthermore, immobilization procedures enhance in many instances operational stabilities of biocatalysts. Among the various methods of cell immobilization, entrapment into polymeric materials has most frequently been used. For example, Escherichia coli cells with high aspartase activity, immobilized in polyacrylamide (ref. 27) or κ-carrageenan (ref. 28), have been used in Japan since 1973 for continuous industrial production of L-aspartic acid from fumaric acid. Under optimized conditions, the aspartase activity of E.coli cells immobilized in κ-carrageenan showed a half life of 693 days ref. 28).

Biotransformations with liquid two-phase systems In their natural environment microbial cells and enzymes display their activities in aqueous media. Since enzymes act on dissolved substrates, the biotransformation of lipophilic substrates is limited by their solubility in the reaction medium. The solubility of lipophilic compounds in water can be improved to a certain extent by the use of detergents or water-miscible solvents. However, higher concentrations of the solubilized substrate, together with the solubility mediators might inhibit or even poison the biocatalyst. Such problems can be circumvented by adding a second, water-immiscible phase, e.g. a water-immiscible organic solvent with high solubility for the lipophilic substrate. Thus, the biocatalyst remains in the aqueous phase, while the organic phase serves as a reservoir for the lipophilic substrate and product. The biotransformation takes place either in the aqueous phase where the substrate concentration is determined by its solubility in water or at the interface between the two

phases. An ideal organic solvent used for this purpose should be immiscible with water, exhibit high solubility for the substrate and product in question and not inhibit the activity of the biocatalyst. Examples of organic solvents which have successfully been used for such liquid two-phase systems are: n-alkanes, cyclohexane, toluene, benzene, chloroform, carbon tetrachloride, methylene dichloride, ethyl or butyl acetate and diethyl or dibutyl ether (refs. 29-32).

In liquid two-phase systems with great excess of the organic phase, the reaction equilibrium of hydrolytic enzymes can be shifted from hydrolysis to condensation. This opens for example the possibility of enzymatic formation of esters, amides, peptides or of enzymatic polymerization of sugars, nucleotides etc. (refs. 33,34).

Biotransformations carried out in liquid two-phase systems offer the following advantages: (1) high concentration of lipophilic substrate/product in the organic phase; (2) continuous slow release of the substrate into the aqueous phase, and withdrawal of the product by the organic phase, maintains the biotransformation and minimizes inhibitory effects on the biocatalyst; (3) easy phase separation facilitates the recovery of the product from the organic phase and the repeated use of the biocatalyst in the aqueous phase.

Isolation of biotransformation products

Biotransformation products contained in the reaction medium are either recovered from the whole broth or from the supernatant after removal of the biomass by filtration or centrifugation. First steps of product isolation aim at the concentration and/or fractionation of the product. Depending on the physical and chemical properties of the product to be recovered and on the chemical composition of the whole broth, the following methods are taken into consideration: extraction, ion exchange, adsorbtion to polymeric resins, precipitation and distillation. The resulting raw product can be further purified by chromatographic techniques, fractionated distillation, crystallization, decolorization and drying. Small product quantities can in many cases be readily obtained by applying preparative gas chromatography or HPLC. An extended review on product purification from biotechnological processes has recently been published by Belter et.al. (ref. 35).

FIELDS OF APPLICATION

Taking advantage of the favourable properties of bio-catalytic processes (see above), biotransformations have been employed to carry out reaction steps which are hardly or not at all feasible by pure chemical methods. Typical synthetic problems which have been solved successfully by biotransformations are the following:

- Introduction of a chiral center into a molecule
- Resolution of racemates
- Selective conversion of a particular functional group among several groups with similar reactivities
- Regioselective functionalization of a specific non-activated carbon
- Reactions under mild conditions

In practical applications, a particular biotransformation step leading from a substrate A to the desired product B usually represents a key reaction among chemical reaction steps in a synthetic pathway leading to a specific target product. Examples will be given below. In other cases, biotranformations are used to bring about any highly specific modification of a relatively complex molecule yielding a new homogeneous product. As an example, drug analogs have been prepared in this way from known pharmaceutically active compounds and subsequently evaluated with respect to their pharmacological activity, toxicity or pharmacokinetics in comparison to the original compounds. Finally, biotransformations have also served as a model for the evaluation of the drug metabolism in mammalian organisms (ref. 36).

Introduction of chiral centers

Most biotransformations described in literature aim at the introduction of optical activity into a molecule. This is achieved either by enzyme catalyzed conversion of a non-chiral substrate into a chiral product or by enantioselective conversion of only one of the enantiomeric forms of a racemic substrate resulting in a resolution of the racemate (see next chapter). Stereoselective biotransformation steps have often been used for redesigning optically active natural products in their nature-identical absolute configuration as well as for the syntheses of various optically active pharmaceuticals which may display totally different activity/toxicity patterns depending on the absolute configuration at their chiral centers. In the following, some selected examples of industrial interest will be discussed.

L-Aspartic acid and L-malic acid by stereospecific condensation of ammonia or water to fumaric acid: The asymmetric addition of NH_3 by Escherichia coli with high aspartase activity (ref. 37) or of H_2O by Brevibacterium flavum with high fumarase activity (ref. 38) to the double bond of fumaric acid (1, Fig. 1) yields optically pure L-aspartic acid (2) or L-malic acid (3), respectively. If desired, L-aspartic acid can be decarboxylated by action of Pseudomonas dacunhae (ref. 39) yielding L-alanine (4).

Both biotransformations of fumaric acid are carried out in Japan as continuous processes on an industrial scale using fixed-bed reactors containing immobilized Escherichia coli or Brevibacterium flavum cells (refs. 37,38). Microbial production of L-alanine from L-aspartic acid has also been established as an industrial process (ref. 39).

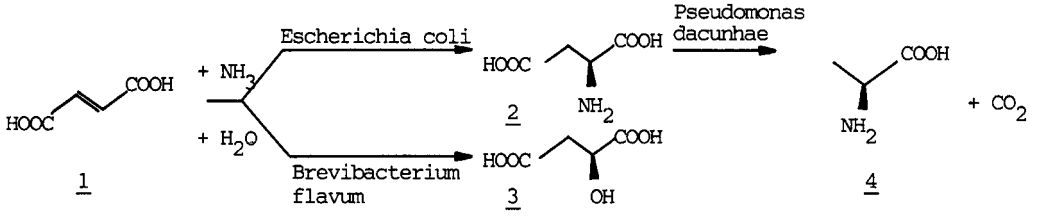


Fig. 1. Asymmetric biotransformations of fumaric acid (1)

L-Phenylalanine by stereospecific condensation of ammonia and trans-cinnamic acid or by reductive amination of phenylpyruvic acid (Fig. 2): L-Phenylalanine (7), a constituent of the dipeptide sweetener aspartame, has been produced by asymmetric addition of NH_3 to the double bond of trans-cinnamic acid (5), catalyzed by phenylalanine ammonia-lyase from Rhodotorula rubra (ref. 40).

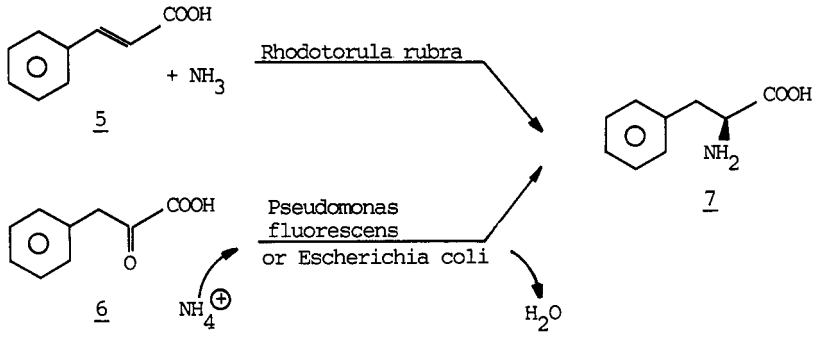
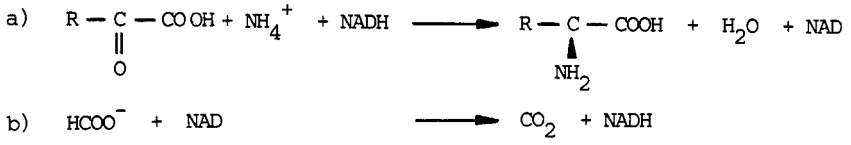


Fig. 2. Production of L-phenylalanine (7) from non-chiral precursors.

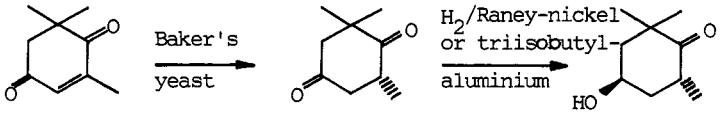
The same product was efficiently obtained by reductive amination of phenylpyruvic acid aminotransferase containing strains of Pseudomonas fluorescens (ref. 41) or Escherichia coli (ref. 42). Both processes have been run continuously using immobilized whole cell reactors and are shown to be cost-effective processes for L-phenylalanine production (refs. 40,41). A series of other L-amino acids have also been produced by reductive amination of the corresponding α-ketoacids using NADH-dependent L-leucindehydrogenase from Bacillus sphaericus in a membrane reactor according to the following scheme (ref. 43)

Scheme 1



The second reaction where formate is oxidized to CO_2 by formate dehydrogenase from Candida boidinii serves merely for the regeneration of the cofactor NADH which was covalently attached to polyethylene glycol (molecular weight 10'000) in order to be retained by the membrane.

A useful building block for the syntheses of optically active 3-hydroxy-carotenoids by stereospecific reduction of oxo-isophorone (8): The following reaction sequence (Fig. 3) has been carried out on a ton scale in order to prepare (4R,6R)-4-hydroxy-2,2,6-trimethylcyclohexanone (10) (ref. 44) which represents an optically active key synthon for the syntheses of natural 3-hydroxy-carotenoids e.g. (3R,3'R)-zeaxanthin, (3R)-cryptoxanthin, (3S,3'S)-astaxanthin and structurally related terpenoid compounds such as the plant growth regulators (6S)-abscisic acid and (3S,5R,6S)-xanthoxin as well as the flavour compounds (3S,5R)-loliolide, (5R)-actinidiolide, (5R)-dihydroactinidiolide, (6S,9S)-theaspirone, (6S)-dehydrovomifoliol, (6S,9R)-blumenol A and picrocrocine (refs. 45,46):



8 9 10

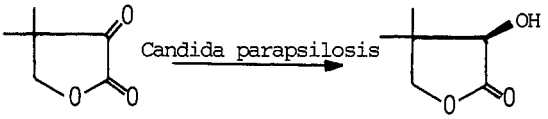
Fig. 3. Synthesis of (4R,6R)-4-hydroxy-2,2,6-trimethylcyclohexanone (10) as a chiral key synthon for the synthesis of optically active carotenoids and related nature-identical products.

In a first step, the double bond of inexpensive oxo-isophorone (8) is stereospecifically reduced with commercially available Baker's yeast yielding the saturated diketone (9) in crystalline and optically pure form. Subsequent chemical reduction of the less hindered ketogroup leads to the desired trans-diastereomeric hydroxy-ketone (10). The chemical reduction is preferably carried out by hydrogenation in presence of a nickel catalyst or with triisobutylaluminium as a reducing agent. Small amounts of the undersired cis-diastereomer can easily be removed by chromatographic methods (ref. 44).

Two further versatile optically active building blocks are (S)-3-methyl-γ-butyrolactone and (S)-2-methyl-γ-butyrolactone. Both have been prepared with high optical purity by stereospecific reduction of suitable unsaturated precursors, catalyzed by Baker's yeast or Geotrichum candidum (ref. 47). They have been used for the synthesis of a variety of optically active compounds, including natural vitamin E. Many other cases of stereospecific microbial reduction of substituted double bonds using predominantly yeasts or fungi have been reported in literature.

Preparation of D-pantolactone (12) by stereospecific reduction of 2-oxo-pantolactone (11):

D-pantothenic acid, has efficiently been prepared by stereospecific reduction of the keto group of 2-oxo-pantolactone (11) according to the following scheme (ref. 48):



11 12

Fig. 4. Stereoselective reduction of 2-oxo-pantolactone (11)

In a broad screening, Candida parapsilosis has been identified as the microorganism providing the desired product with highest concentration and highest chemical and optical yield. Other microorganisms have been observed, which produce almost exclusively the opposite L-enantiomer or mixtures of both enantiomers (ref. 49).

In a similar fashion a series of other ketones has successfully been reduced to optically active alcohols. In most cases yeasts or fungi served as biocatalysts.

Microbial transformations leading to optically active 3-hydroxy-2-methylpropionic acid (17): Optically active 3-hydroxy-2-methylpropionic acid has been prepared with the aid of several types of biotransformations (Fig. 5):

a) Stereospecific hydroxylation of isobutyric acid (13) by Pseudomonas putida yields (S)-3-hydroxy-2-methylpropionic acid (ref. 50).

b) Stereospecific hydration of the double bond of methacrylic acid (14) yields the (S)- or (R)-configuration of 3-hydroxy-2-methylpropionic acid, depending on the selected microorganism (ref. 51).

c) Enantioselective oxidation of prochiral 2-methyl-1,3-propanediol (15) with Gluconobacter roseus yields (R)-3-hydroxy-2-methylpropionic acid (ref. 52).

d) Stereoselective reduction of ethyl α-formylpropionate (16) with Candida humicola yields (R)-3-hydroxy-2-methylpropionic acid (ref. 53).

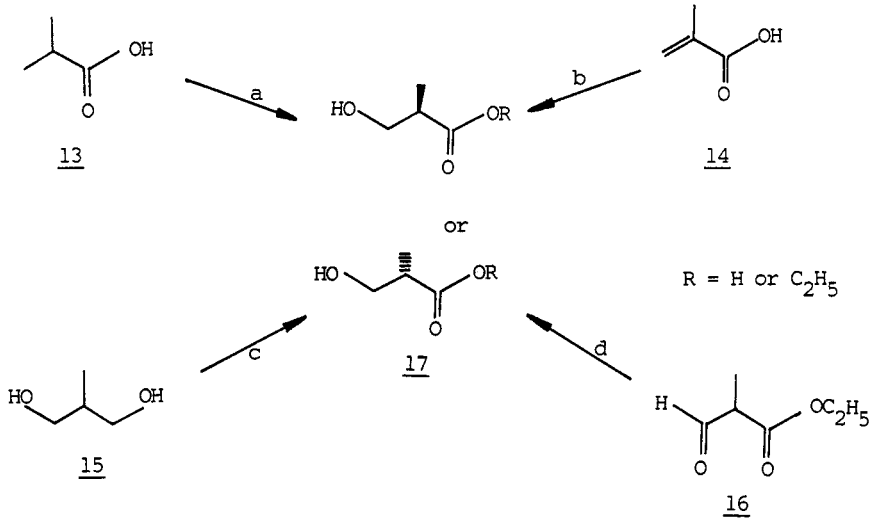


Fig. 5. Different microbial transformations leading to (S)- or (R)-3-hydroxy-2-methylpropionic acid

Optically active 3-hydroxy-2-methylpropionic acid has been utilized as a useful bifunctional starting material for the syntheses of innumerable optically active products such as, natural vitamin E, lasalocid A, rifamycin S, monoensin and captopril (see literature cited in ref. 53).

Asymmetric C-C-bond formation: Asymmetric condensation of benzaldehyde (17) and acetaldehyde (18) is mediated by the yeast Saccharomyces cerevisiae (ref. 54). The condensation product is (R)-1-hydroxy-1-phenylpropan-2-one (19) which serves as an intermediate in the synthesis of (1R,2S)-ephedrine (20), a natural alkaloid from ephedra plants with interesting pharmacological activities.

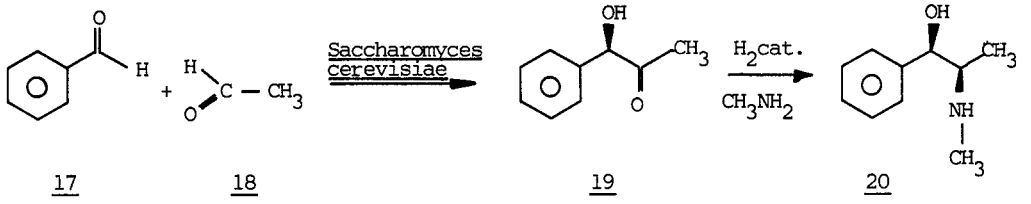


Fig. 6. Synthesis of (1R,2S)-ephedrine (20)

The R-configured center of asymmetry introduced by the microbial condensation induces the 2S-configuration at the new chiral center which arises as a result of the reductive addition of methylamine (performed by chemical methods).

Another example for asymmetric C-C bond formation is the addition of HCN to aldehydes mediated by oxynitrilase, an enzyme which is readily available from almonds and which accepts a broad range of aldehydes as substrates (ref. 55). The resulting optically active cyanohydrins can be chemically converted to chiral α-hydroxyacids, aminoalcohols or acyloins.

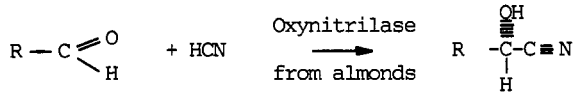


Fig. 7. Formation of optically active cyanohydrins by oxynitrilase

Resolution of racemates

The ability of enzymes to discriminate between enantiomers of a racemic mixture has been frequently employed for the resolution of racemates, a task which is difficult to achieve by chemical methods. The maximum yield of the desired enantiomer in a resolution process with 2 enantiomers is limited to 50 %. As it is usually not acceptable to lose the remaining half of the racemic substrate, the undesired enantiomer has to be recycled by racemisation. In most cases hydrolytic enzymes (hydrolases) are used to carry out optical resolution by enantioselective hydrolysis; typical substrates are racemic esters, amines, amides, lactones, N-acyl-compounds, hydantoins etc. In general, hydrolases are easy to handle, since they do not require any cofactors, exhibit good stability and accept a broad variety of substrates. Several useful hydrolases are commercially available and have been widely used for optical resolutions on a preparative scale. Examples are pig liver esterase, chymotrypsin, trypsin, subtilisin, papain and lipases from different origin (ref. 1). However, in many cases it is still necessary to search for suitable microorganisms harbouring a tailor-made enzyme which is able to hydrolyze a given substrate with high enantiomeric specificity. Several industrially significant examples will be given below.

Resolution of racemic amino acid derivatives: L-aminoacylase from Aspergillus oryzae has successfully been used for the enantioselective hydrolysis of various racemic N-acyl amino acids (ref. 56). This enzyme liberates the L-amino acid while the remaining acyl-D-amino acid can easily be recycled by racemization (Fig. 8a). L-Aminoacylase from Aspergillus oryzae immobilized on DEAE-Sephadex has been used since 1969 for the industrial manufacture of L-alanine, L-methionine, L-phenylalanine, L-tryptophan and L-valine on a 1000 l scale (ref. 57). A D-amino-acylase has been found in Streptomyces olivaceus (ref. 58). Another enzyme, namely hydantoinase (dihydropyrimidase), catalyses the enantioselective ring cleavage of racemic amino acid hydantoins, which are available cheaply via the Bucherer synthesis, yielding optically acitve N-carbamoyl amino acids. The carbamoyl moiety can easily be removed in presence of nitrite under acidic conditions yielding the optically active amino acid. This reaction can also be carried out enzymatically by means of a carbamoylase (ref. 59). The remaining enantiomer of the amino acid hydantoin can be recycled by racemization under alkaline conditions (Fig. 8b).

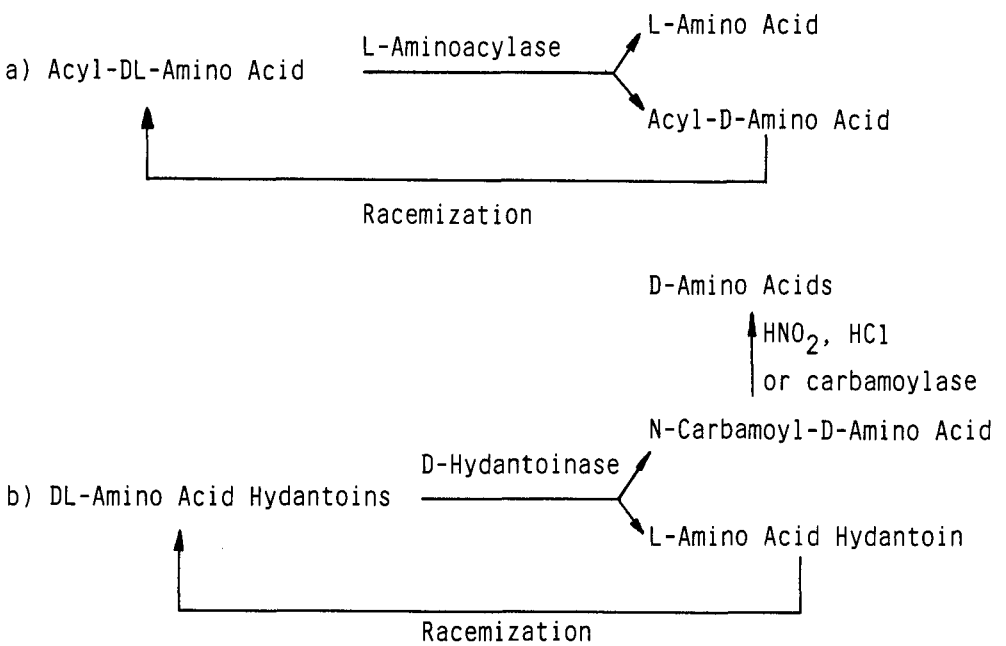


Fig. 8. Enantioselective hydrolysis of Acyl-DL-amino acids or DL-amino acid hydantoins by L-aminoacylase or D-hydantoinase

Hydantoinases with D-specificity are widely destributed among microorganisms and they usually accept a relatively broad spectrum of substrates (ref. 60). D-specific hydrolysis of racemic amino acid hydantoins has been applied to the production of various D-amino acids which are valuable constituents of semisynthetic penicillins or cephalosporins.

On the other hand, a L-specific hydrolase from Bacillus brevis has been described and employed for the production of L-glutamic acid from the corresponding DL-hydantoin (ref. 61). A bacterium isolated from soil was found to be suitable for the manufacture of a L-hydantoinase and a carbamoylase which hydrolyzes the N-carbamoyl-L-tryptophan under retention of the absolute configuration (ref. 62). An analogous process has recently been developed with a strain identified as Flavobacterium sp. (ref. 63). Up to 50 g of L-tryptophan could be obtained per liter of culture broth per day. Since the D-enantiomer was spontaneously racemized and recycled under slightly alkaline fermentation conditions (pH 8,5), the conversion yield from DL-5-indolyl-methylhydantoin was almost 100 % (ref. 63).

A further industrially significant process using enantioselective hydrolysis of an amino acid precursor is the manufacture of L-lysine from DL-2-aminohexane-6-lactam which is synthetically available from cyclohexanone. The yeast Cryptococcus laurentii hydrolyzes selectively the L-form of the racemic substrate and liberates L-lysine (ref. 63). The remaining D-form can be enzymatically racemized by a racemase from Achromobacter obae (ref. 64). Thus, the racemic substrate can be converted almost quantitatively into L-lysine in a one-stage process employing both microorganisms simultaneously (Fig. 9, ref. 65).

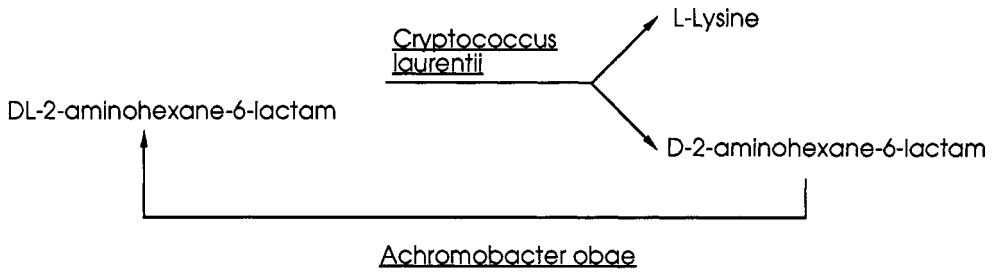


Fig. 9. Production of L-lysine from DL-2-aminohexane-6-lactam.

Resolution of racemates using esterases or lipases. Stereospecific ester hydrolyses using esterases or lipases from various sources provide a powerful method for the preparation of chiral esters, carboxylic acids or alcohols by resolution of the corresponding racemic ester. Useful enzymes which exhibit hydrolytic activity and enantiomeric specificity with a broad structural range of racemic esters are chymotrypsin, trypsin, subtilisin, pig liver esterase and pig pancratic lipase (ref. 1).

Lipases turned out to be a particularly useful category of enzymes; they accept a broad spectrum of substrates, including lipophilic substances and in addition they are relatively stable in aqueous and even in organic media, where lipophilic substances can readily be processed. For example, porcine pancreatic lipase is stable for several hours in toluene or decanol even at 100° C (ref. 67). In addition, lipases may display a totally different substrate spectrum in organic media (ref. 68) or - even more important - catalyse reactions that are hardly possible in water, such as transesterification, esterification or aminolysis (ref. 69). High regioselectivity and stereoselectivity have been observed with lipases catalysing transesterifications or esterifications in organic media. For example, yeast lipase or porcine pancreatic lipase catalyse highly stereospecific esterification or transesterifications in nearly anhydrous organic solvents. In this manner, a number of optically active alcohols, carboxylic acids and their esters have been produced on a preparative scale (ref. 70).

Regioselective conversion of a functional group among several groups with similar reactivities

In contrast to chemical methods, enzymes can differentiate between several functional groups of similar reactivity within the same molecule. They convert regioselectively only one of the available functional groups, affording a homogeneous product. The classical example is the biotransformation step used for more than 50 years in the technical synthesis of vitamin C (ref. 71), namely the conversion of D-sorbitol to L-sorbose which is performed at high substrate concentration nearly quantitatively by Acetobacter suboxydans (Fig. 10). Among the 6 hydroxy groups of the substrate molecule, microbial oxidation takes place exclusively in position 2, yielding L-sorbitol without any by-product.

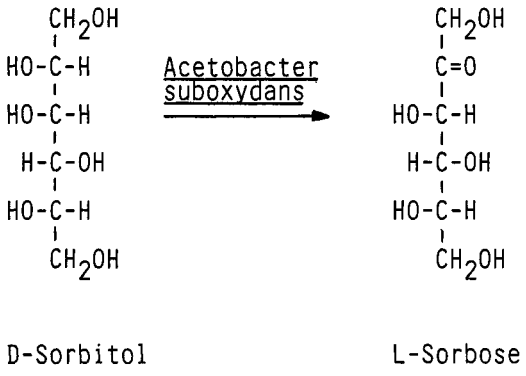


Fig. 10. Regioselective oxidation of D-sorbitol

Many alternative regioselective biotransformations have been proposed for vitamin C synthesis (review: ref. 72). Recently an elegant procedure yielding 2-oxo-L-gulonic acid (the immediate precursor of vitamin C) from D-glucose has been described. In a first regioselective bioconversion mediated by Erwinia sp. glucose is oxidized to 2,5-dioxo-gluconate which is subsequently reduced regioselectively by Corynebacterium sp. yielding pure 2-oxo-L-gulonic acid (ref. 73). By cloning the 2,5-dioxo-gluconate reductase from Corynebacterium sp. in Erwinia herbicola, a strain has been constructed which converts D-glucose directly to 2-oxo-L-gulonic acid (ref. 74).
Regioselective oxidation of glycerol, yielding dihydroxyacetone, by Acetobacter strains and isomerization of glucose to fructose (doubled sweetening power) are two further examples from carbohydrate chemistry (ref. 75). Glucose isomerase is widely distributed among microorganisms (ref. 76). Furthermore, regioselective acylation of carbohydrates and related compounds (e.g. formation of monobutyryl esters) has been achieved by protease (subtilisin)-catalysed transesterification in anhydrous dimethylformamide as the reaction medium (ref. 77).

Lipase from Chromobacterium viscosum and protease from Bacillus subtilis (subtilisin) are capable of regioselective esterification of dihydroxy steroids in dry acetone. Interestingly, the model compound 5-α-androstane 3ß, 17ß-diol was exclusively esterified at the OH-group in 3-position with the lipase, while subtilisin showed marked preference for the C-17 OH-group (ref. 78).

Regioselective functionalization of a certain non-activated carbon

By means of enzymatic catalysis functional groups can be regioselectively introduced at a specific non-activated position in a substrate molecule, which is not attacked by chemical reagents. This is impressively illustrated by the regioselective hydroxylation of steroids: depending on the microorganism used, nearly every carbon atom in the steroid skeleton can be regioselectively and even stereoselectively hydroxylated. Regioselective introduction of a double bond by enzymatically catalysed dehydrogenation is also feasible. Besides hydroxylations and dehydrogenations many other microbial modifications of steroids have been described in the literature and compiled in useful reviews (refs. 7,79,80). Since the hormonal and other biological activities of steroids depend on their proper functionalization, microbial transformations represent a unique tool in the synthesis of highly valuable pharmaceuticals from readily available steroid raw materials such as deoxycholic acid (from animal bile), stigmasterol (from soy beans) and diosgenin (from Mexican Diascorea plants).

For example, the antiinflammatory activity of corticosteroids is dependent on an oxygen function at the 11-position in the steroid skeleton, which is introduced in an industrial process by microbial 11-hydroxylation (Fig. 10).

Starting from Reichstein's compound S which is readily accessible by chemical degradation of diosgenin or stigmasterol, cortisol is obtained by 11ß-hydroxylation with Curvularia lunata (ref. 81). Cortisone is obtained from cortisol by chemical oxidation. Subsequent 1-dehydrogenation of cortisol or cortisone with Arthrobacter simplex yields prednisolone or prednisone respectively. These 1-dehydro derivatives of the natural cortical hormones exhibit markedly increased antiinflammatory activity. Another antiinflammatory agent, triamcinolone (9α-fluoro-16α-hydroxyprednisolone), is produced by 16 α-hydroxylation of 9α-fluoro-cortisol mediated by Streptomyces roseochromogenes (ref. 82). Subsequent 1-dehydrogenation yielding triamcinolone can again be achieved by means of Arthrobacter simplex.

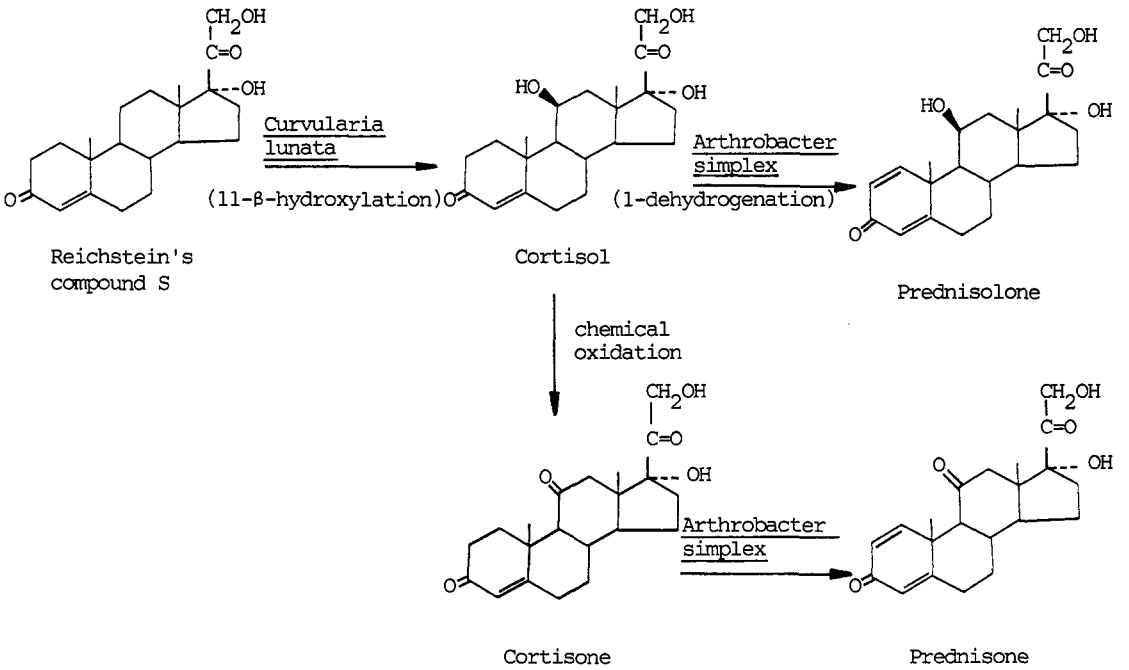


Fig. 11 Preparation of Cortisol and Cortisone via microbial 11ß-hydroxylation of Reichstein's compound S. Further microbial 1-dehydrogenation leads to Prednisolone or Prednisone.

Mild reaction conditions

As outlined above, biotransformations take place under mild reaction conditions and are therefore suitable for the conversion of labile molecules. For example, the hydrolytic cleavage of the side chain of penicillin G is preferably carried out by means of enzymatic catalysis. The appropriate enzyme, penicillin-acylase, is found in Escherichia coli and many other microorganisms (ref. 83). The hydrolysis product, 6-aminopenicillanic acid serves as the starting material for the preparation of many useful semi-synthetic penicillins. Enzymatic hydrolysis is superior to chemical hydrolysis, since the mild reaction conditions (together with the regioselectivity of the enzyme) guarantee a highly selective hydrolysis of the amide bond in the side chain, without hydrolytic opening of the ß-lactam ring which would destroy antibiotic activity.

Some chemical processes require harsh reaction conditions which demand relatively large amounts of energy and may impair the environment. If an alternative biotechnological production process exists at all, it is often hampered by economical aspect, althought it might be advantageous with respect to environmental protection. A recent example realized in Japan exemplifies the substitution of a well-established large-scale chemical process by a biotransformation, namely the hydration of acrylonitrile yielding acrylamide. Acrylamide, a precursor of various polymers, is a commodity chemical produced worldwide in an amount of about 100'000 tons/year. Depending on the procedure used, the chemical hydration of acrylonitrile is carried out in presence of 85 % sulfuric acid at a temperature of 90° C, followed by neutralization with ammonia or by passing an acrylonitrile solution over a Raney copper catalyst at about 100° C (ref. 84). On the other hand, a novel process has recently been developed involving nitrile hydratase from Pseudomonas chloraphis (refs. 85,86) or Rhodococcus sp. (refs. 86,87). Nitto Chemical Industry, Japan, is using this biotransformation process for the manufacture of acrylonitrile on a 4000 tons/year scale since 1987 (refs. 84,87). A similar enzymatic hydration process with a potential for industrial large-scale application is the production of nicotinamide from 3-cyanopyridine mediated by nitrile hydratase from Rhodococcus rhodochrous (ref. 88).

CONCLUSIONS AND PROSPECTS

Biotransformations can be a useful tool in organic chemistry, particularly in cases where a product is difficult to obtain by conventional chemical methods, e.g. complex molecules including asymmetric centers and multiple functional groups. Successful design of combined chemical/biotechnological syntheses requires close collaboration of specialists from both

disciplines. If a new biotransformation has to be developed as a part of a novel reaction sequence, the microbiologist has usually to carry out an extensive screening in order to identify the appropriate microorganism. Subsequent biotechnical development work includes:

- optimization of biomass cultivation and biotransformation conditions (medium, temperature, pH, pO_2, agitation, substrate feeding regime etc.)
- strain improvement by classical methods or by genetic engineering
- process engineering, i.e. design and construction of an appropriate production facility
- elaboration of an efficient product isolation procedure
- minimization of the production costs
- scaling up

The chemist has to supply the substrate to be subjected to biotransformations, he may be involved in analytical work and in product purification and finally he is responsible for the chemical conversion of the biotransformation product into the desired target molecule. With respect to the vast number of useful biotransformations reported in the literature, only relatively few have so far been applied on an industrial scale. The following reason might account for this situation: a) many chemists are reluctant to introduce biotechnological steps in their production processes or they are not even aware of the opportunities offered by biotransformations; with improved interdisciplinary education and in view of an increasing number of successful examples, this situation is starting to change; b) expensive and lengthy research and development work necessary to establish a biotransformation process may be deterrent; c) many biotransformations competing with chemical processes suffer from high process costs due to limited stability and/or activity of the biocatalyst or from low product concentrations due to solubility or toxicity problems. Such problems will increasingly be solved by the rapid development of useful new technologies such as:

- immobilization techniques which improve the stability (longevity, reusability) of biocatalysts and render continuous production processes possible
- water immiscible organic solvents as reaction media; these increase the solubility of substrates and can protect biocatalysts which prefer aqueous environments from toxic effects
- recombinant DNA techniques which can be used to increase the production of the enzyme responsible for the desired biotransformation
- protein engineering (e.g. site-directed mutagenesis) which can help to increase the stability and/or improve the catalytic properties of the enzyme in question, or even to tailor an enzyme for specific purposes.

Skilful application of these techniques in combination with conventional development methods will contribute to cost reductions and render further industrial biotransformation processes feasible and attractive, also from an economical point of view. Furthermore, increasing pressure of environmental constraints will favour processes which can be run under mild conditions, even if they are not superior from the economical point of view.

Syntheses involving two or more sequential biotransformation steps will become increasingly feasible as a one-stage process. This can be achieved by simultaneous action of two (or several) microorganisms in the same reactor (ref. 65) or by using recombinant DNA technology to clone the genes for the necessary enzymes into a single microorganism (ref. 74).

Further progress in the purification and immobilization of enzymes as well as in cofactor regeneration in vitro will promote the technical application of purified enzyme reactors.

In view of the nearly unlimited reservoir of different enzyme activities existing in nature and the exciting achievements of modern biotechnology there is still an enormous potential for further progress in the field of biotransformations. It is expected that biotransformations will be increasingly exploited as a useful and often unique tool in organic chemistry.

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